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13. ABSTRACT *(Maximum 200 Words)*

Many of the molecular events that control normal development of the mammary gland are the same events that, when misregulated, result in cancer. Therefore, the understanding of normal developmental processes in the mammary gland is a crucial first step to rational design of therapeutics which target these systems in breast cancer.

The Wnt family of genes, which were first identified for their role in mouse mammary tumorigenesis, initiate a signaling cascade that manifests in the stabilization of β-catenin protein. The proposed experiments are based on the hypothesis that misregulation of this pathway results in an accumulation of stabilized β-catenin, and genes involved in growth, cell death, and cell invasion are upregulated inappropriately, resulting in tumorigenesis.

The proposed experiments use two complimentary strategies to study β-catenin's direct role in mammary gland development and tumorigenesis, gain- and loss-of-function experiments. Reconstitution experiments using cells expressing stabilized β-catenin and transgenic mice expressing a dominant negative mutant β-catenin ($\beta\text{-cat}^{DN}$) specifically in the mammary gland provide opposite approaches for study. These two systems will be used to analyze changes in morphology, downstream signaling, and functional differentiation, comparing gain- and loss-of-function of β-catenin.

Many factors implicated in mammary oncogenesis regulate β-catenin, and β-catenin is clearly involved in tumorigenesis in other organ systems, suggesting a role for β-catenin in mammary oncogenesis. The proposed experiments will characterize β-catenin's direct role in mammary gland development and tumorigenesis.

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Introduction

β-catenin's involvement in Wnt and other signaling pathways and its oncogenic potential in other tissues strongly suggest a role for β-catenin in mammary tumorigenesis. This study proposes to examine the direct role of β-catenin in normal mammary gland development and tumorigenesis through gain- and loss-of-function experiments.

Body

Task 1: To study β-catenin's involvement in mammary gland development and carcinogenesis in virgin and pregnant mice using a primary culture reconstitution method

The first Annual Report summarized the difficulties experienced using the mammary reconstitution method. This dilemma prompted a discussion within our group regarding enriching the primary culture for multi-potent progenitor cells. We followed this idea and developed a method for isolating a population of progenitor cells; these experiments are described in Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM and Goodell MA, "Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population", Developmental Biology, in press (Appendix B).

As the manuscript describes, the mammary gland side population (MG-SP) cells represent the purest population of mammary progenitor cells. However, technical limitations of the method restrict the further characterization of these cells due to very low cell viability. The manuscript also describes an alternative isolation method, using expression of Stem cell antigen-1 (Sca-1) as a marker of progenitor cells. The long-term plan is to retrovirally infect these purified populations with exogenous genes and perform reconstitution experiments. These experiments are ongoing within the lab and will be continued by other students.

Since the mammary reconstitution method has proven to be so problematic, we have pursued alternative methods for studying β-catenin's involvement in virgin and pregnant mammary development. Specifically, stabilized β-catenin is expressed in virgin mammary glands by using an *in vitro* Cre-lox system. Mammary glands are isolated from animals that carry lox sites flanking exon 3 of β-catenin. Primary culture cells are isolated from these glands, grown in culture, and infected with adenovirus expressing the Cre recombinase (Adeno-Cre). Upon recombination, the cells and all their future progeny express stabilized β-catenin protein. These cells are then injected into cleared fat pads as previously described, allowed to reconstitute a mammary gland, and analyzed for morphological and molecular phenotype. These ongoing experiments will offer a much more efficient and reliable method to study β-catenin's role in development and carcinogenesis than the original methods described.

Task 2: To characterize β-catenin's signaling function using a dominant negative transgenic model

Tasks 2a-b: Completed and described in first Annual Summary

Four lines of transgenic mice were developed which express a dominant negative form of β-catenin (β-cat^{DN}) specifically in the mammary gland. Two of these lines express β-cat^{DN} under the Mouse Mammary Tumor Virus (MMTV) promoter, while two lines use the Whey Acidic Protein (WAP) promoter. Both of these promoters express transgene specifically in the mammary gland during pregnancy and lactation, and all four lines have the same morphological phenotype.

Task 2c: Characterize mammary gland phenotype by whole mount and histochemical analysis

Morphological analysis of transgenic glands during development was conducted by whole mount and histological analysis. Glands were isolated from transgenic and non-transgenic littermates throughout mid-late pregnancy (days 10-18) and at 1-day lactation. In addition, immunohistochemical methods were used to correlate transgene expression with morphological phenotype.

These experiments demonstrated that β-cat^{DN} expression could be detected at day 10 of pregnancy (10P) through day 13 of pregnancy (13P), but expression later than 13P is rarely detected. The expected expression pattern based on other transgenic models using the same promoters predicted transgene expression beginning at 10P and continuing throughout lactation.

In contrast, morphologically, the 10-13P glands appeared normal compared to wild-type littermates. Later in pregnancy and lactation, the glands showed a reduction in lobuloalveolar development and less epithelium overall. This disjointed pattern of early transgene expression and late morphological phenotype led to the following hypothesis: transgene expression either induces a decrease in proliferation or an increase in apoptosis or both, and this early event manifests itself later in development as a decrease in lobular development.

Task 2d: Characterize downstream targets

The lack of lobular development in transgenic animals suggests that these glands either have a decrease in proliferation or an increase in apoptosis or both during development. To test this hypothesis, transgenic and non-transgenic glands were analyzed at days 10-13 of pregnancy, a time when the normal mammary gland is highly proliferative and rates of apoptosis are extremely low. This time point also correlates with transgene expression.

Bromodeoxyuridine (BrdU) incorporation was utilized to measure proliferation in mammary glands from transgenic mice and wild type littermates. These experiments found a two- to three-fold decrease in proliferation in transgenic glands compared to non-transgenic glands. The terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) method was employed to analyze apoptotic cells in these glands. Transgenic glands at day 10-13 of

pregnancy display a five-fold increase in apoptosis compared to non-transgenic glands. In addition, immunofluorescence experiments provide correlative data that the cells expressing transgene are not proliferating but are often undergoing apoptosis. These experiments demonstrate that the downstream effects of expression of the dominant negative β-catenin (β–cat^{DN}) construct are a decrease in overall rate of proliferation and an increase in apoptosis,

These proliferation and apoptosis data were verified in cell culture experiments, using the HC11 mammary epithelial cell line. Cells were retrovirally infected with stabilized β-catenin (gain-of-function) or β-cat^{DN} (loss of function) constructs. Like in the *in vivo* experiments, cells were analyzed for BrdU incorporation to measure proliferation and by the TUNEL assay to detect apoptosis. These experiments demonstrated no change in proliferation of cells infected with either gain-of-function or loss-of-function β-catenin constructs. Cells infected with β-cat^{DN} (loss-of-function) construct showed a five-fold increase in apoptosis compared to uninfected cells. This apoptosis data correlates with that obtained in the transgenic *in vivo* experiments. Experiments are currently ongoing which will increase our understanding of the molecular mechanism underlying this apoptotic phenotype. A manuscript describing these data is in preparation.

Key Research Accomplishments

- Isolated for the first time a population of functional mammary epithelial stem/progenitor cells
- Initiated experiments using adenoviral-mediated recombination of floxed β-catenin primary culture cells and transplantation *in vivo*
- Established the critical importance of Wnt signaling in normal lobular development
- Confirmed the importance of the Wnt/β-catenin signaling pathway in regulating proliferation as well as its additional function in regulating apoptosis

Reportable Outcomes

Manuscript:

- Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM and Goodell MA, "Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population", Developmental Biology, in press

Poster:

- National Cancer Institute Mouse Models of Human Cancers Consortium, Washington DC, January 2002

Presentations:

- National Cancer Institute Mouse Models of Human Cancers Consortium, Washington DC, January 2002
- Baylor College of Medicine Graduate Student Symposium, October 2001
- 54^{th} MD Anderson Cancer Center Symposium on Fundamental Cancer Research, October 2001

Methods developed:

Isolation and purification of a population of mammary stem/progenitor cells has been accomplished, based on methods and molecular marker expression which are novel to the mammary gland.

Transgenic mice:

Four lines of transgenic mice were established which express β-cat^{DN} under mammary-specific promoters and have severe defects in development and lactation.

Collaborations/funding opportunities:

A valuable collaboration was established with Margaret A. Goodell, Ph.D. and together the group applied for and received a Concept Award from Department of Defense Breast Cancer Research Program.

Conclusions

Experiments that were initiated as a means of improving a method to be used in Task 1, eventually yielded very interesting results independent of the original motivation. The manuscript in press describes the isolation of a population of mammary epithelial cells which are enriched for the ability to reconstitute a mammary gland. This is the first time that functional mammary stem cells have been isolated from the total population of cells in the mammary gland. The transgenic experiments have also yielded very interesting data, including the first conclusive evidence that the Wnt/β-catenin signaling pathway is absolutely required for normal lobular development. Additionally, while much work has focused on β-catenin's involvement in proliferation, we have identified a somewhat unexpected function in regulating apoptosis. These experiments have all contributed to breast cancer research by increasing our understanding of the normal development of the mammary gland, a very important step in understanding the molecular misregulation that leads to cancer.

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Appendix B:
Manuscript in press
Developmental Biology

Sca-1^{pos} Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population

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Abstract

Mammary epithelium can functionally regenerate upon transplantation. This renewal capacity has been classically ascribed to the function of a multipotent mammary gland stem cell population, which has been hypothesized to be a primary target in the etiology of breast cancer. Several complementary approaches were employed in this study to identify and enrich mammary epithelial cells that retain stem cell characteristics. Using long-term BrdU labeling, a population of label retaining cells (LRCs) that lack expression of differentiation markers has been identified. LRCs isolated from mammary primary cultures were enriched for stem cell antigen-1 (Sca-1) and Hoechst dye-effluxing "side population" properties. Sca-1^{pos} cells in the mammary gland were localized to the luminal epithelia using Sca-$1^{+/GFP}$ mice, were progesterone receptor negative, and did not bind peanut lectin. Finally, the Sca-1^{pos} population is enriched for functional stem/progenitor cells, as demonstrated by its increased regenerative potential compared to Sca-1^{neg} cells when transplanted into the cleared mammary fat pads of host mice.

Keywords: mammary, stem cells, BrdU, Sca-1, label retention, progesterone receptor.

Introduction

The mammary gland provides a unique model for the study of growth, differentiation, apoptosis, and pattern formation involved in developmental processes. A characteristic of the mammary gland is its ability to postnatally proliferate, terminally differentiate and involute upon successive cycles of pregnancy, lactation and involution, respectively. Mammary gland growth and differentiation share many mechanistic similarities with developmental processes occuring during organogenesis in the mouse embryo, including the regulation of patterning through growth factor signaling pathways. Mammary epithelium is also capable of completely and functionally regenerating upon transplantation. This impressive renewal capacity has been ascribed to the function of a multipotent mammary gland stem cell population (For review see (Smith and Chepko, 2001)). Thus, the postnatal mammary gland can be utilized for the analysis of developmental pathways not only involved in the regulation of growth and patterning, but also in the determination of cell lineages and differentiation.

The existence of mammary gland stem cells was first demonstrated through classical transplantation studies conducted by DeOme (DeOme et al., 1959). DeOme and colleagues observed that epithelium isolated from several different regions of the mammary gland at any stage of postnatal development was capable of generating functional mammary epithelial outgrowths containing ductal, lobuloalveolar and myoepithelial cells. Subsequent studies by Daniel indicated that mammary epithelium could be serially transplanted, but exhibited senescence following seven or eight transplants (Daniel et al., 1968). Kordon and Smith suggested, through the use of retroviral-tagging, that progeny from a single cell can give rise to a complete mammary gland upon transplantation (Kordon and Smith, 1998). These data indicate that mammary gland stem cells are distributed throughout the gland, and exhibit a potent yet

limited self-renewal capacity. Furthermore, the mitotic and self-renewal capacities of mammary gland stem cells are thought to make them highly susceptible to mutagenesis, thus making them the primary targets in the etiology of breast cancer.

A stem cell population would be expected to contribute to the cell types in mammary gland development through symmetric cell division (for self-renewal) and asymmetric cell division (to generate progenitors) (Chepko and Smith, 1997). During ductal morphogenesis (3-8 weeks of age in the mouse), the mammary gland is rapidly proliferating and contributing to cell lineages in the developing epithelium. At the terminal ends of the ducts are bulb-like structures called terminal end buds (TEB). TEBs are the proliferating units of the mammary gland, and as they penetrate the fat pad, their progeny make up the luminal and myoepithelial cell types of the gland. At the distal tip of the TEB exists a layer of highly proliferative cells termed "cap cells" that lack steroid receptors and are thought to represent an undifferentiated, stem/progenitor cell population, giving rise to differentiated luminal and myoepithelial cells in the subtending ducts (Daniel and Silberstein, 1987; Williams and Daniel, 1983). In the mature animal, the mammary gland remains in a quiescent state until the onset of pregnancy. During this period, stem cells are thought to be scattered throughout the gland and contribute to the maintenance of the ductal epithelial network.

Several morphologically distinct cell populations have been identified in the mammary gland. These cell types are distinguished based on DNA condensation, size, shape, location, cytoplasmic granularity, and nuclear morphology using electron microscopy. These distinctions have been used to detect a population of cells termed "small light cells" (SLC), which make up about 3% of the total epithelial component in rat mammary glands. SLCs exhibit morphological characteristics of putative undifferentiated, division-competent mammary gland stem cells

(Chepko and Smith, 1997; Smith and Chepko, 2001). These studies demonstrated that the mammary gland consists of a heterogenous population of epithelial cells in different stages of differentiation. However, few molecular markers have been identified that are expressed during mammary gland differentiation, and the analysis of mammary epithelial cell lineages has been limited. Furthermore, the lack of cell surface lineage markers in the mammary gland has resulted in the inability to characterize the abiltiy of purified epithelial cell populations to regenerate mammary gland outgrowths.

This study describes for the first time the enrichment of a cell population within primary mouse mammary epithelial cell (MEC) cultures that displays multipotent mammary gland progenitor/stem cell characteristics. BrdU label-retention, FACS analysis, and transplantation methods have been combined to enrich and characterize these cells and to determine their functional capacity for regeneration. In addition, these studies have increased our understanding of the largely unknown differentiation lineage during virgin mammary gland development.

Materials and Methods

BrdU Label Retention

Alzet minipumps #2002 (Durect Corp., Cupertino, CA) were filled with 200 µl of 60 mg/ml BrdU (Sigma #B-5002) (designed to release 0.5 µl/hr for 14 days). Three-week-old C57B6 female mice (Taconic, Germantown, NY) were anesthetized with Avertin and a minipump was implanted interscapularly into each animal. Fourteen days later, the animals were anesthetized, and the pumps were removed. This label retention experiment was conducted four times, using 19-24 mice in each experiment. Inguinal glands from mice were collected and BrdU was analyzed by immunofluorescence at week 0 (4 glands), week 9 (8 glands), and intervening weeks (2 glands each). After the 9-week chase period, lymph nodes were removed from #4 inguinal glands, and the glands were digested into mammary epithelial primary culture suspension (DeOme et al., 1959; Pullan and Streuli, 1996). The cells were sorted by FACS for the MG-SP population and by magnetic column for Sca-1 expression (as described below). These sorted cells were then spun onto glass slides, fixed with cold acetone for 10 min, and washed with PBS. Label retaining cells on these slides were identified by submerging in 0.07N NaOH for 2 min, washing in PBS pH 8.5, staining with anti-BrdU-FITC (Becton Dickinson #347583) for 1 hr, and counterstaining with DAPI (Vector #H-1200). The number of $BrdU^{pos}$ cells in each of these populations was counted and graphed.

Immunofluorescence Localization

Glands were fixed in 4% paraformaldehyde for 2 hr on ice, embedded in paraffin, and cut into 5 µm sections. After deparaffinization, antigen retrieval was performed as previously described (Seagroves et al., 2000). Sections were incubated with primary antibody overnight at room

temperature in a humidity chamber, washed in PBS and incubated with secondary antibody for 1 hr at room temperature. Sections were then washed 1 hr in PBS and mounted in mounting medium containing DAPI (Vector #H-1200). Primary antibodies used were: mouse monoclonal anti-BrdU-FITC (Becton-Dickinson #347583), rabbit-anti-human PR (Dako #A0098), mouse monoclonal anti-human cytokeratin 18 (Progen #61028, clone Ks 18.04), and rabbit-anti-mouse cytokeratin 14 (Covance #PRB-155P). Secondary antibodies used were: goat-anti-rabbit-Texas Red (Molecular Probes #T6391) and goat-anti-mouse-Texas Red (Molecular Probes #T6390).

SP Sorting

All ten mammary glands were dissected out of mature virgin B6;129S-Gtrosa26 mice (Jackson Labs), and the epithelial cell fraction was isolated as described previously (Pullan and Streuli, 1996). Cells were plated on plastic tissue culture dishes in growth medium consisting of F12 (Gibco BRL) supplemented with insulin (5 μg/ml; Sigma), hydrocortisone (1 μg/ml; Sigma), epidermal growth factor (10 ng/ ml; Gibco BRL), penicillin/streptomycin (100 μg/ml; Gibco BRL), gentamycin (50 μg/ml; Sigma), and fetal bovine serum (10%; JRH Biosciences). After 72 hr in culture, the cells were trypsinized with 0.05% trypsin/0.02% EDTA (JRH Biosciences), washed twice in Hanks' Balanced Salt Solution (HBSS; Gibco BRL), and stained with a final concentration of 5 μg/ml Hoechst-33342 in Dulbecco's Modified Eagle's Medium (DMEM) with 2% FBS at 37°C for 90 min as described previously (Goodell et al., 1996). Sca-1 expression in the MG-SP cells was analyzed by staining the cells with anti-Sca-1-PE antibody (BD Pharmingen #553336, clone E13-161.7) for 15 min on ice following the Hoescht-33342 treatment. Analysis and sorting were performed on a triple laser MoFlo (Cytomation, Fort Collins, CO). The Hoechst dye was excited at 350 nm and its fluorescence was measured at two

wavelengths, 450/20 BP filter Blue and 675 EFLP optical filter Red, as described previously (Goodell et al., 1996).

SP Cell Injections

Collected SP cells were washed with HBSS, and $2.5x10^4$ or $7.5x\ 10^4$ cells were mixed with $2x10^5$ unsorted wild type C57BL/6 cells per injection site. The inguinal glands of 21-day old Rag-$1^{-/-}$ females (Jackson Labs) were cleared of endogenous epithelium as previously described (DeOme et al., 1959). Cells were injected into the cleared fat pads of these mice using a 50 µl Hamilton syringe in a blind method. Cells were allowed to grow out for 6 weeks, and then the animals were bred to induce lobuloalveolar development. At day 10-16 of pregnancy, glands were surgically removed and stained for lac-Z expression as previously described (Rijnkels and Rosen, 2001).

Sca-1 Magnetic Sorting

Enrichment of Sca-1-expressing cells was achieved by sorting cells using the MACS system (Miltenyi Biotec, Sunnyvale, CA). Whole primary culture cells isolated from mature virgin C57BL6 mice (Harlan Sprague Dawley) were incubated with biotinylated anti-Sca-1 antibody (PharMingin #553334) for 10 min on ice, washed in $DMEM^+$ (DMEM with 2% fetal bovine serum (JRH Biosciences #12106-500M) and 10 mM HEPES), incubated with streptavidin-conjugated microbeads (Miltenyi Biotec #130-048-101) for 5 min on ice, incubated with streptavidin-PE (Molecular Probes #S-866) for 5 min on ice, washed with $DMEM^+$, and loaded onto a MACS column (Miltenyi #130-041-306). The column was set up in a Miltenyi magnet so the magnetized microbeads and all cells that adhered to them would be retained on the column.

The flow through was collected as the Sca-1^{neg} fraction, and the Sca-1^{pos} cells were eluted from the column by removing the column from the magnetic field and washing with $DMEM^{+}$. For the injection experiments, this process was repeated; each fraction was loaded on new columns, and the purity of the Sca-1 enrichment and viability of the cells (by propidium iodide staining) was analyzed on a FACSCAN (Becton Dickinson, Sunnyvale, CA).

Sca-1-Bead Sorted Cell Injections

Mammary epithelial primary cultures were isolated from #4 and #5 mammary glands (without the lymph node) of mature virgin C57BL6 mice (Harlan Sprague Dawley) as described above. Cells were sorted on the MACS system and Sca-1^{pos} and Sca-1^{neg} fractions were collected and analyzed for purity and viability (by propidium iodide staining). Recipient C57BL6 females at 21 days of age were cleared of endogenous mammary epithelium, and either 10,000 or 50,000 viable Sca-1^{pos} or Sca-1^{neg} cells were injected into each cleared fat pad in a blind method. After 4 weeks, a subset of the animals were bred, and at day 8-10 of pregnancy the injected fat pads were surgically removed and fixed in 4% paraformaldehyde for 2 hr on ice. Whole mounts were prepared as previously described (Williams and Daniel, 1983) and images were captured using Olympus dissecting microscope and Sony video camera (#DXC-151A).

Sca-$1^{+/GFP}$ Transplants

Mammary glands were removed from mature Sca-$1^{+/GFP}$ mice and cut into 2-3 mm pieces. One piece of tissue was transplanted into each cleared fat pad of 21-day old Rag-$1^{-/-}$ females (Harlan Sprague Dawley). After 8 weeks, the fat pads containing transplanted tissue and subsequent outgrowths were harvested and fixed as described below.

Sca-1$^{+/GFP}$ Fluorescence

Transplanted mammary outgrowths and entact Sca-1$^{+/GFP}$ mammary glands were surgically removed, fixed in 2% paraformaldehyde for 2 hr, frozen in Tissue Freezing Medium (Triangle Biomedical Sciences #H-TFM) on dry ice, and sectioned in 60 μm sections. Sections were incubated with Texas Red-X phalloidin (Molecular Probes #T-7471) or rabbit-anti-human PR (Dako #A0098) and anti-rabbit-Texas Red (Molecular Probes #T6391) as described above except incubation times were increased to 1 hr/10 μm of section. Sections were analyzed using Zeiss 510 laser scanning confocal microscope.

Sca-1$^{+/GFP}$ Sorted Cell Injections

Mammary epithelial primary cultures were isolated from inguinal and pelvic mammary glands (without the lymph node) of mature virgin C57BL6/129 Sca-1$^{+/GFP}$ mice as described above. Cells were sorted on a Beckman Coulter Altra FACS machine with an argon laser tuned to 488 nm. GFP fluorescence was measured at 525 nm BP filter, GFP^{pos} and GFP^{neg} fractions were collected in DMEM with 2% FBS, and data was analyzed using Expo 32 software. Between 1,000 and 10,000 viable Sca-1^{pos} or Sca-1^{neg} cells were injected into each cleared fat pad of Rag-1$^{-/-}$ females as described above. After six weeks, the transplanted fat pads were surgically removed and whole mounts were prepared as described above.

Results

BrdU Label Retention in Mammary Epithelial Cells

In an effort to identify quiescent stem cells in the mammary gland, a bromodeoxyuridine (BrdU) label-retention approach was employed that has been used successfully to identify stem cells in the skin (Lavker and Sun, 1982), cornea (Cotsarelis et al., 1989), and hair follicle (Cotsarelis et al., 1990; Taylor et al., 2000). BrdU was administered continuously to mice undergoing ductal morphogenesis when stem cells are proliferating and should, therefore, incorporate the thymidine analog into their DNA. The labeling period was followed by a 9-week chase period during which BrdU retention was monitored via immunofluorescence using a FITC-conjugated anti-BrdU antibody. During the chase, ductal proliferation will continue until the ducts reach the edge of the mammary fat pad. At the end of ductal morphogenesis, the mammary gland will remain relatively quiescent, and stem cells are expected to exhibit lower proliferative and apoptotic indices than more differentiated cell types. Under these conditions, quiescent stem cells should retain BrdU while the proliferating and terminally differentiated cell types will lose the label either following mitosis or after undergoing apoptosis.

Specifically, a continuous dose of BrdU was administered to 3-week old female mice for 14 days by means of an interscapularly implanted Alzet minipump (Fig. 1A). After the 2-week BrdU dose, minipumps were removed and mammary gland biopsies were taken every week for nine weeks to monitor label-retaining cells. Approximately 70% of MECs were labeled during the 2-week dose of BrdU (Fig 1B,F). At the end of the 9-week chase period, less than 5% of luminal epithelial cells retained BrdU (Fig.1C,F), and these cells were termed label-retaining cells (LRCs). After the chase period, LRCs ranged in intensity from brightly labeled (C, arrows)

to dimly labeled (C, arrowheads), suggesting that LRCs represent a spectrum of cells that have undergone varying numbers of cell divisions.

To characterize the differentiation status of LRCs in the mammary gland, MECs were co-stained for expression of BrdU and either keratin (K)14/18 or the progesterone receptor (PR). After the 2-week dose with BrdU, approximately 40% of $BrdU^{pos}$ cells also expressed PR (Fig. 1D, F). Throughout the chase period the number of co-localizing cells steadily decreased to approximately 1.5% after nine weeks (Fig 1E,F). The total percentage of PR^{pos} cells did not significantly change during this time period, which is consistent with previous reports (Seagroves et al., 2000). A population of LRCs was detected which did not express K-14 or -18, markers of the myo- and luminal epithelium, respectively (Fig. 1G,H,I,J; arrows). Additionally, a sub-population of label-retaining luminal cells did express K14/18 (arrowheads), which correlates with a model in which label retaining cells represent a spectrum of quiescent stem cells and early differentiating cells. Thus, these data demonstrate that a population of LRCs does not express common mammary gland differentiation markers, suggesting they are maintained in a less differentiated state.

MG-SP Cells in the Mammary Gland

The efficient efflux of the fluorescent dye Hoechst-33342 has been demonstrated previously to be a mechanistic characteristic of pluripotent hematopoietic stem cells, (Goodell et al., 1996) . In those experiments, bone marrow cells were treated with Hoechst dye and then analyzed by FACS at two different emission wavelengths. A small, distinct population of bone marrow termed "side population" (SP) cells effluxes the Hoechst dye. Further, these SP cells contain the entire hematopoietic potential of whole bone marrow, establishing their functional

capacity as hematopoietic stem cells (Goodell et al., 1996). SP cells with multipotent stem cell characteristics also have been identified in other regenerative tissues, such as muscle (Gussoni et al., 1999; Jackson et al., 1999) and liver (G. Wulf, M. Goodell, submitted). Additionally, SP cells from bone marrow and muscle are enriched for the expression of stem cell antigen-1 (Sca-1), a cell surface protein shown to be a marker of pluripotent hematopoietic stem cells (Goodell et al., 1996; Jackson et al., 1999; Spangrude et al., 1988).

A similar SP cell staining protocol was employed to isolate potential mammary gland stem cells from primary MEC cultures. Mammary glands were isolated from mice and manually and enzymatically digested as previously described (DeOme et al., 1959; Pullan and Streuli, 1996). These cells were then stained with Hoechst dye and analyzed by FACS. Similar to bone marrow, the mammary gland contained a distinct population of Hoechst-effluxing SP cells, referred to as MG-SP cells (Fig. 2A). The MG-SP population represented approximately 2-3% of the total population of epithelial cells in the mammary gland, which is consistent with previous estimates of the percentage of stem cells in the mammary gland (Chepko and Smith, 1997). Likewise, the same previous report predicted mammary stem cells to be small with little cellular complexity; forward- and side-scatter plots confirmed MG-SP cells also have these characteristics (data not shown). Treatment with verapamil, a multi-drug transporter inhibitor shown to eliminate the SP population in bone marrow, also reduced the MG-SP population by four-fold (data not shown). Thus, the mammary gland contains a population of cells which efflux Hoechst dye in a manner similar to that described previously for hematopoietic and muscle stem cells.

Analysis of MG-SP Cells

Since Stem cell antigen-1 (Sca-1) is expressed on functional hematopoietic stem cells, it was hypothesized that Sca-1 might also be expressed by the MG-SP cells. Sca-1 is a GPI-anchored protein frequently utilized in murine hematopoietic stem cell enrichment strategies (Spangrude et al., 1988). It is encoded by Ly-6A/E, a member of the Ly-6 superfamily of highly homologous genes conserved from humans to snake (Mao et al., 1996; Ploug and Ellis, 1994). Sca-1 is expressed on some differentiated cell types, including in the kidney and brain, and on T cells, where it has been implicated as a costimulatory molecule (Malek et al., 1986; Miles et al., 1997; Stanford et al., 1997; van de Rijn et al., 1989). Since Sca-1 is expressed on both bone marrow- and muscle-derived SP cells (Goodell et al., 1996; Gussoni et al., 1999; Jackson et al., 1999), its expression on MG-SP cells was characterized.

Primary MECs were incubated with Hoechst dye, stained with an anti-Sca-1 antibody, and then analyzed by FACS. About 20% of the total population of the MECs and 75% of the MG-SP cells were found to be Sca-1^{pos} (Fig. 2B,C). These data suggest that the MG-SP population is enriched in Sca-1^{pos} cells.

To minimize the possibility of contamination from blood and lymphatic cells (since Sca-1 is expressed on some hematapoietic cells), only inguinal and pelvic mammary glands that were devoid of lymph nodes were used to make primary cultures. Expression of hematapoietic cell markers was analyzed in the MG-SP and Sca-1^{pos} populations to determine if these cells resulted from blood contamination. MG-SP cells did not express c-Kit or CD45, markers of hematopoietic stem cells and peripheral blood (Fig 2C and data not shown). In addition, the Sca-1^{pos} population in total MEC culture was found to be largely CD45 and lineage marker negative (Fig 2D and data not shown). Similar MG-SP and Sca-1^{pos} populations were also

isolated from primary MECs after culturing cells for 5 days with daily media changes (data not shown), indicating that the MG-SP and Sca-1^{pos} cells were epithelial in origin and were not derived from blood contamination.

Label Retention in SP Cells

To determine the proliferative nature of the MG-SP cells, the presence of LRCs was analyzed in this population. Primary MECs were isolated from mice that received the long BrdU label followed by the 9-week chase. These cells were then FACS sorted for MG-SP cells and $BrdU^{pos}$ cells were determined by immunofluorescence. A four-fold increase in LRCs in the MG-SP population was detected as compared to the non-SP population (Fig.2E). These data suggest that MG-SP cells proliferate during ductal morphogenesis, but remain either more quiescent or less apoptotic than the non-SP population in the mature mouse mammary gland, consistent with their candidacy as mammary gland stem cells.

Outgrowth Potential of Purified MG-SP Cells

To determine if MG-SP cells are capable of repopulating the mammary gland, MG-SP cells were isolated and transplanted into cleared mammary fat pads in limiting numbers. In this experiment, MECs were isolated from B6;129S-GtROSA26 (ROSA) donor mice and cultured for five days. Cells were cultured in this experiment in an effort to improve their viability through the SP-staining and sorting procedure. Cultured MECs retained a MG-SP profile similar to fresh MECs (data not shown). MG-SP cells from the ROSA primary cultures were then isolated by FACS and limiting numbers of cells were mixed with $2x10^5$ wild type C57BL6 primary MECs. This competitive repopulation protocol was used to ensure the presence of any

paracrine interactions between MG-SP and non-MG-SP cells that may be required for proper outgrowth. The mixed cell populations were transplanted into the cleared fat pads of Rag-$1^{-/-}$ recipient mice. Immuno-compromised recipients were used to prevent host rejection of cells derived from the ROSA mixed background. Recipient mice were bred after six weeks to induce lobuloalveolar development in the mammary gland, and outgrowths were isolated two weeks later. Whole mounts of outgrowths were stained with X-gal to detect β-galactosidase (β-gal) activity, an indication that such cells originated from the ROSA MG-SP cells. Patches of β-gal-expressing cells could be detected in outgrowths from as few as $2.5x10^4$ transplanted ROSA MG-SP cells (data not shown), and robust staining was detected in outgrowths from $7.5x10^4$ transplanted ROSA MG-SP cells (Fig. 2F,G). In this whole mount, two distinct outgrowths were detected by X-gal staining (Fig. 2F, arrowheads). Higher magnification revealed β-gal-positive cells in both ductal (Fig. 2G, arrowheads) as well as alveolar epithelium (Fig. 2G, arrows). These experiments indicate that purified MG-SP cells have mammary gland outgrowth potential and can contribute to alveolar and ductal epithelial populations.

Previous studies have shown that at least 100,000 total MECs must be injected into the fat pad to reliably form a mammary outgrowth (Smith, 1996). While the current experiments demonstrate outgrowth potential from 25,000 - 75,000 injected MG-SP cells, the combination of proteinase and Hoechst dye treatment and high pressure FACS involved in isolating MG-SP cells resulted in <5% viability (data not shown). Therefore, we estimate that <4,000 viable MG-SP cells were injected and resulted in outgrowth (Fig 2F,G). These results suggest that there was a significant enrichment in outgrowth potential in the MG-SP population as compared to total primary MECs.

Thus, several characteristics of the MG-SP cell population support a stem/progenitor nature of these cells: 1) MG-SP cells comprise a constant 3-5% of the total epithelial population in virgin and pregnant mice; 2) they proliferate when the mammary gland is rapidly establishing the ductal network; 3) they remain quiescent in the mature mouse; and 4) when transplanted, their progeny can develop into ductal and alveolar cell types. However, the low viability of MG-SP cells restricted the use of this approach to further characterize mammary stem cells. Since the MG-SP population was found to be enriched in Sca-1^{pos} cells, Sca-1 expression in primary MECs was investigated as an alternative method to enrich for a population of stem/progenitor cells.

Localization and Characterization of Sca-1^{pos} Cells in the Mammary Epithelium

The localization of Sca-1-expressing cells in the mammary gland was analyzed using a targeted GFP insertion into the Sca-1 locus (Sca-$1^{+/GFP}$). These mice are heterozygous for a mutation in which an EGFP cassette is inserted into the Sca-1 locus, placing it under the regulatory control of the Sca-1 promoter. These mice express GFP in a temporal and spatial manner similar to that observed for Sca-1 in hematopoietic cell lineages, and although this mutation disrupts the endogenous Sca-1 allele, Sca-1 haploinsufficiency has no important consequences for hematopoiesis (T. Graubert et al, submitted).

Frozen sections of Sca-$1^{+/GFP}$ glands were analyzed by confocal microscopy, and Sca-1-GFP expression was detected scattered throughout the ductal luminal epithelium (Fig. 3A-D). Pieces of mammary tissue from Sca-$1^{+/GFP}$ donor mice also were transplanted into the cleared fat pads of wild type recipient mice, and the resulting outgrowths were harvested 8 weeks later. The images shown in Fig. 3A&B were derived from these outgrowths of transplanted Sca-$1^{+/GFP}$

tissue. Even following proliferation and differentiation resulting from transplantation, Sca-1-GFP is expressed in the same pattern in these outgrowths as endogenous Sca-1-GFP. Interestingly, more intense GFP expression was detected specifically at the distal tips of growing ducts (Fig. A&B), with decreased expression also detected scattered throughout mature ducts (Fig. 3A-C). Presumably, this difference in expression level may be attributed to the long half-life of GFP, which may be 1 to 2 days. Thus, these observations of Sca-GFP expression (intense at the distal tips of growing ducts and scattered sporadically throughout the luminal epithelium) are consistent with the expected distribution of stem or progenitor cells, as predicted from transplantation experiments (Kordon and Smith, 1998).

In an effort to characterize the differentiation status of these Sca-GFP cells, expression of PR and interaction of these cells with peanut lectin were analyzed (Fig. 3 D&E). Immunofluorescence experiments revealed that Sca-1-GFP and PR expression observed in mature ducts did not co-localize in the same cells (Fig. 3D). Likewise, FACS analysis of these Sca-1-GFP^{pos} cells showed essentially no overlap with cells binding peanut lectin (Fig. 3E), a marker of differentiation in the mammary gland which appears to interact with MUC4 on mammary epithelial cells (Li et al., 2001; Rudland, 1992). These data suggest that the Sca-1^{pos} cells, even those in the mature duct, represent a population of less differentiated cells.

Sorting Mammary Epithelial Cells Based on Sca-1 Expression

The apparent relationship of MG-SP, LRCs and Sca-1^{pos} cells suggested that it might be possible to isolate mammary stem cells with improved viability using their Sca-1^{pos} status as a criterion for selection (Fig 4). Accordingly, an immunoaffinity technique was employed using an anti-Sca-1 antibody conjugated to microbeads via a biotin/streptavidin interaction. Freshly

prepared primary MECs, isolated from inguinal mammary glands void of lymph nodes (to reduce muscle and lymphocyte contamination), were incubated with biotinylated anti-Sca-1 antibody and streptavidin-conjugated beads. These were then applied to a column in a magnetic field. The Sca-1^{pos} cells that adhered to the magnetic column were washed and then eluted by removing the column from the magnetic field. FACS analysis verified that this method depleted Sca-1^{pos} cells in the flow-through fraction but enriched for a Sca-1^{pos} population in the eluate (Fig. 4A-C). The bound cell fraction (Fig. 4C) exhibited a 3.5-fold increase in Sca-1^{pos} cells, while the depleted population (Fig. 4B) displayed a 3.5-fold decrease in Sca-1^{pos} cells, as compared to the starting MEC population (Fig. 4A).

To determine the presence of LRCs in the Sca-1^{pos} population, primary MECs were isolated from mice following long term BrdU labeling and a 9-week chase period. These cells were sorted based on Sca-1 expression using the magnetic cell sorting technique. Following purification, Sca-1^{pos} and Sca-1^{neg} cells were cytospun onto coverslips, stained for BrdU immunofluorescence, and manually counted. Approximately 19% of purified Sca-1^{pos} cells retained BrdU after the 9-week chase as compared to 7-8% of the cells in the Sca-1-depleted population (Fig. 4D), a greater than two-fold enrichment. These data were confirmed by BrdU immunofluorescence and FACS analysis of Sca-1-enriched and Sca-1-depleted populations (data not shown). Thus, Sca-1^{pos} cells isolated from mouse mammary gland primary cultures contain an enriched population of growth quiescent cells, a characteristic consistent with their role as putative stem/progenitor cells.

These data demonstrate that both the MG-SP and Sca-1^{pos} populations contain a subpopulation of quiescent cells (LRCs) with low turn-over rates. However, since the MG-SP cells contain a four-fold enrichment in LRCs while Sca-1^{pos} cells are only enriched two-fold, this

suggests that the MG-SP cells are less proliferative than the Sca-1^{pos} cells. One possible model to explain these results is that Sca-1 is expressed in a broader population of cells that contain subpopulations of both MG-SP and LRCs (see Fig. 6). This subpopulation of Sca-1^{pos}/MG-SP/LRC cells may represent a less proliferative, more primitive population of progenitor/stem cells.

Outgrowth Potential of Sca-1-Enriched Cells

To characterize the regenerative potential of Sca-1^{pos} cells, transplantation experiments into cleared fat pads of syngeneic host mice were again employed using the Sca-1-enriched and Sca-1-depleted populations of freshly prepared primary MECs (Fig. 5A). Cells were sorted using two rounds of magnetic enrichment, and purity reached 86% Sca-1 ^{pos} and 92% Sca-1^{neg} cells in the enriched and depleted fractions, respectively. Either 10,000 or 50,000 cells were injected into each cleared fat pad, and after 6 weeks the outgrowths were removed from the mice and stained as whole mounts with hematoxylin to visualize the epithelium. The extent of outgrowth was defined as partial outgrowth (ductal structures fill 5-50% of the fat pad; Fig. 5B), full outgrowth (ductal structures fill >50% of the fat pad; Fig. 5C), or no epithelial outgrowth. All Sca-1-enriched injections formed a partial or full outgrowth, while only 4 out of 10 Sca-1-depleted injections formed any outgrowth (Fig. 5A).

To achieve higher purities, a complementary approach was taken, using low pressure FACS sorting of primary MECs isolated from Sca-1$^{+/GFP}$ mice. Using this FACS-based method, the GFP^{pos} population was enriched to >90% positive, while the depleted population was 99% pure (Fig. 5A). Between 1,000 and 10,000 cells were injected into each cleared fat pad, and outgrowths were harvested after six weeks. All six injections of GFP^{pos} cells resulted in an

outgrowth, including a minimum of only 1,000 or 2,000 cells injected. Of the six GFP^{neg} injection sites, none formed an outgrowth (Fig. 5A). These data complement the Sca-1-bead sorting technique, offering a purer population of Sca-1^{pos} cells and subsequent increased outgrowth potential. Thus, Sca-1^{pos} cells isolated from mammary gland primary cultures appear be enriched for a population of multipotent progenitor cells and contain increased outgrowth potential. Most significantly, depletion of the Sca-1^{pos} cells from a population removes outgrowth potential from that population.

Since the Sca-1-enriched cells produced a morphologically normal outgrowth, it was expected that all three epithelial cells types (luminal, myoepithelial, and alveolar cells) should be present in these outgrowths, as interactions between these cells with their environment is required for normal mammary gland growth and differentiation. To this end, sections of the whole mounted outgrowths were stained with hematoxylin and eosin (H&E) and analyzed by light microscopy to study the morphology of Sca-1-enriched outgrowths. Outgrowths derived from GFP^{pos} cells contained normal luminal and myoepithelial cells as well as TEBs with distinct cap and body cells (Fig. 5D, E, F), indicative of normal ductal morphogenesis (Williams and Daniel, 1983). Additionally, outgrowths derived from GFP^{pos} cells formed normal alveolar buds during early pregnancy (Fig. 5D,F). These data suggest that Sca-1^{pos} cells isolated from primary MEC culture have the potential to differentiate into multiple mammary epithelial cell types.

Discussion

In this study, several complementary approaches have been employed to identify and, for the first time, enrich mammary epithelial cells that retain stem cell characteristics. Long term BrdU label retention studies, FACS sorting for an MG-SP population, and Sca-1 enrichment all use differing criteria to identify cohorts of mammary epithelial cells with overlapping stem cell characteristics. These populations of cells collectively represent a spectrum of undifferentiated and differentiated cells with unique molecular and regenerative characteristics.

Differentiation in the Mammary Gland

Cell lineage and early differentiation markers are largely uncharacterized in the mammary gland, unlike many other model systems of development. While decades of work have analyzed milk protein production as a marker of functional differentiation during pregnancy and lactation, few markers of differentiation during virgin development exist and these remain controversial. Here, the identification of Sca-1 as a marker of undifferentiated cells contributes not only to our understanding of stem/progenitor cells in the mammary gland, but also contributes to the body of knowledge regarding differentiation in the mammary gland.

In this study, two populations of cells, identified independently, share characteristics of progenitor/stem cells and remain less differentiated than their neighbors. Neither the LRCs nor the Sca-1^{pos} cells express PR, which has been shown to be expressed in non-proliferating, differentiated mammary epithelial cells (Brisken et al., 1998; Russo et al., 1999; Seagroves et al., 2000). In the current study, however, administration of BrdU for two weeks identified >40% of cells that divided during the labeling period also expressed PR, but after the chase period, <5% of $BrdU^{pos}$ cells expressed PR. Taken together, these data suggest that PR expressing cells do

not undergo proliferation, yet cells that have undergone proliferation are capable of eventually expressing PR. Although the exact lineage of differentiation in the mammary gland is unknown and controversial, we propose that proliferating progenitor cells can give rise to more differentiated cells which then exit the cell cycle and remain quiescent.

Mammary Epithelial Sca-1^{pos} Cells Have Increased Outgrowth Potential

The Sca-1$^{+/GFP}$ outgrowths demonstrated that a highly pure population of Sca-1^{pos} cells retained outgrowth potential (100%) compared to a Sca-1-depleted population (0%). In this experiment, as few as 1,000 Sca-1^{pos} cells were necessary to form an outgrowth. Since theoretically, only one stem cell is required to reconstitute an entire gland, injecting 1,000-10,000 cells may not appear to be a significant enrichment. However, unlike hematopoietic cells, which survive naturally in suspension, mammary epithelial cells exist in tissue organization and require paracrine interactions associated with cell-cell adhesion. Therefore, a single mammary stem cell injected into a fat pad would undoubtedly fail to grow out due to the lack of requisite signals and support provided from neighboring epithelial cells. Previous studies have shown that >100,000 total cells must be injected into the fat pad to produce a mammary outgrowth in about 60% of transplant sites (Smith, 1996). Thus, these experiments demonstrate two points: 1) when injecting a highly purified population of Sca-1 ^{pos} cells, 10-100x fewer cells (compared to whole cell population) can be used to achieve consistent outgrowth, and more importantly, 2) depletion of the Sca-1 ^{pos} population removes outgrowth potential.

Smith and colleagues have demonstrated that at least three multipotent cell populations with distinct outgrowth potential exist in the mammary gland. These three multipotent cell populations include cells capable of 1) complete mammary gland outgrowth, 2) ductal

morphogenesis, or 3) lobuloalveolar development (Chepko and Smith, 1997). The current study found that Sca-1 ^{pos} cells retain the ability to contribute progeny to all cell types required to produce a normal epithelial outgrowth. However, the Sca-1 ^{pos} population could represent a homogenous, multipotent population or a heterogeneous population of more committed progenitor cells. Further dilution experiments will be necessary to distinguish between these possibilities, by determining clonality of the outgrowths, and by analyzing senescence in serial transplants.

Relationship of Mammary Epithelial Cells

Several decades of mammary gland research have demonstrated the regenerative capacity of the mammary gland, verifying the presence and potency of mammary gland stem and progenitor cells (Daniel et al., 1968; DeOme et al., 1959; Hoshino and Gardner, 1967). Stem cells in the mammary gland are expected to contribute to all mammary gland cell types including luminal, myoepithelial and alveolar. The progeny from mammary gland stem cells should not only maintain the ductal networks in a mature animal but also produce precursor cells capable of differentiation. This implies that the mammary gland consists of a mixed population of cells within a spectrum of differentiation states. Data presented here demonstrate the presence of several populations in the mammary gland with different regenerative capabilities and molecular markers that characterize these populations. Mammary gland Sca-1^{pos} cells make up about 20% of the total epithelial population, have increased outgrowth potential, are enriched in LRCs and MG-SP cells and are PR negative (Fig. 6). Sca-1^{neg} cells make up about 80% of the epithelial population, have few LRCs, have decreased outgrowth potential and express differentiation markers PR and K18.

From these results, at least three cell populations in the mammary gland can be defined: PR^{pos}, Sca-1^{pos} and MG-SP cells. These results suggest that Sca-1^{pos} and MG-SP cells are not mutually exclusive and represent overlapping populations (Fig. 6). The PR^{pos} cells (and peanut lectin/MUC 4 cells), however, are excluded from the Sca-1^{pos} and presumably the MG-SP populations. The label retention technique demonstrated that the MG-SP and Sca-1^{pos} cells proliferate during ductal development, but are quiescent in the mature animal. Characteristics of these populations make them potential candidates for mammary epithelial stem/progenitor cells.

Confocal microscopy revealed some of the GFP positive cells appeared to contact both the basement membrane and the lumen. Interestingly, previous EM studies have shown that the small light cells (SLC) have limited contacts with the lumen and are primarily basally located (Chepko and Smith, 1997). Since Sca-1^{pos} cells consistently comprised about 20% of the total population of the mammary epithelium and exhibited contact with both the lumen and basement membrane, it is unlikely that these cells exclusively represent the SLC population. Rather, it is possible that the SLCs comprise a subpopulation of the larger Sca-1^{pos} population; likely candidates for the SLC subpopulation are the LRC/MG-SP cells. Recent identification of the functional ABC transporter (brcp1/ABCG2) in SP cells (Zhou et al., 2001) may now provide an alternative method by which to isolate MG-SP cells, avoiding the toxicity problems experienced with the Hoechst dye-staining and sorting protocol.

In the mammary gland, MG-SP cells represent a morphologically homogenous population of small cells with low cytoplasmic to nuclear ratios and decreased forward and side scatter profiles (data not shown), suggesting that these cells do not retain specialized secretory or metabolic function. The low percentage of these cells found in the mammary gland and their distinct morphological properties make MG-SP cells prime candidates for a primitive stem cell

population in the mammary gland. However, further analysis of the capacity of MG-SP cells to clonaly expand into complete mammary glands upon transplantation will be required to further define the stem cell qualities of this population

Stem Cells and Breast Cancer

The unique replicative capacity and clonal expansion of stem and progenitor cells makes these cells susceptible to transformation; it is likely that mutations occurring during stem cell proliferation may by perpetuated throughout the mammary gland, potentially leading to carcinogenesis. This model is supported by several lines of research: 1) in rat DMBA breast cancer models, the targets of carcinogen-induced transformation are the TEBs (the prospective site of stem cells during ductal morphogenesis), and 2) stem cell markers have been shown to be upregulated in human breast cancer (Dulbecco et al., 1986; Russo et al., 1982; Smith and Chepko, 2001; Smith et al., 1990). Previous studies have found that Sca-1 is upregulated in carcinoma cell lines (including mammary lines), and higher levels of Sca-1 correlated with more aggressive, tumorigenic cell lines (Cohn et al., 1997; Katz et al., 1994; Treister et al., 1998). Preliminary data also suggest that mammary epithelial tumor cell lines yielding more aggressive tumors express higher levels of Sca-1 than less aggressive tumor cell lines (B. Welm, S. Tepera, J. Rosen, D. Medina, unpublished observation). Therefore, given the suggestion that Sca-1^{pos} cells may be targets of transformation, it will be interesting to further characterize the status of Sca-1 expression in various breast cancer models.

In summary, this study is the first to report the isolation of functional mammary stem/progenitor cells. While the existence of stem cells in the mammary gland has been previously proven by transplantation studies, the isolation of this population has remained

elusive until now. The availability of this isolated population should permit the identification of additional markers that can be used to follow cell lineages during normal mammary development and the progression of breast cancer. The purification of this population can also be used as a tool to efficiently produce genetically modified outgrowths. Finally, studies are in progress to compare the similarities and/or differences between these mammary progenitor cells with those isolated from other tissues, as well as to analyze their reciprocal tissue plasticity.

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Figure Legends

Figure 1. Long Term BrdU Label Retention

(A) A schematic representation of the experimental design. Three-week old female mice received a continuous dose of BrdU for two weeks via a subcutaneously implanted pump. Upon removal of the pump, mammary gland biopsies were taken each week during the 9-week chase period. After 9 weeks, mammary glands were analyzed for label-retaining cells (LRCs), a small population of quiescent epithelial cells. (B-E) Immunofluorescence analysis of BrdU-labeled cells at week 0 (B,D) and week 9 (C,E). Luminal epithelium was extensively labeled with BrdU at week 0 (B), but only about 5% of luminal epithelium retain this label in week 9 (C). Double immunofluorescence analysis (BrdU-FITC and PR-Texas Red) revealed colocalization of $BrdU^{pos}$ and PR^{pos} cells at week 0 (D), but very little colocalization by week 9 (E, arrows). Quantitation of PR^{pos} and $BrdU^{pos}$ cells throughout the chase period (F) showed constant numbers of PR^{pos} cells, decreasing numbers of $BrdU^{pos}$ cells, and decreasing colocalization of PR and BrdU. (G-J) Colocalization of LRCs with cytokeratin 14- and cytokeratin 18-expressing cells after 9-week chase (BrdU-FITC (G), K14-Texas Red and K18-Texas Red (H), BrdU and K14/18 (I), and DAPI (J)). Two distinct populations of LRCs expressed K14/K18 (arrowheads) and did not express these markers of differentiation (arrows), suggesting LRCs represent a spectrum of differentiated cells. Scale bars represent 100 μm (B-E) and 10 μm (G-J).

Figure 2. Mammary Gland Contains a Pluripotent Population of MG-SP Cells

FACS analysis of Hoechst-33342 exclusion reveals a population of cells which effluxes Hoechst dye (A). These cells represent approximately 3% of total mammary epithelial cells and were termed MG-SP cells. This MG-SP population was enriched for expression of Sca-1 (C) when compared to Sca-1 expression in total MECs (B). $Sca\text{-}1^{pos}$ cells isolated from total MEC culture do not express the

peripheral blood marker CD45 (D). When MG-SP cells were isolated from glands following BrdU incorporation and a 9-week chase, the MG-SP population was enriched for LRCs four-fold compared to the non-MG-SP cells (E). (F and G) Competitive recombination experiments using MG-SP cells isolated from ROSA mammary glands. MG-SP cells, injected into cleared fat pads of host mice, reconstituted an epithelial outgrowth. X-gal staining (indicating the outgrowth originated from ROSA MG-SP cells) revealed two clonal outgrowths (F, arrowheads). Higher magnification (G) illustrates X-gal staining in both ductal (arrowheads) and alveolar (arrows) cell types, suggesting MG-SP cells maintained the ability to differentiate into multiple cell lineages in the mammary gland. Scale bars represent 1 mm.

Figure 3. Expression of Sca-1 in the Mammary Gland

Mammary glands were removed from Sca-$1^{+/GFP}$ mice, and pieces of gland were transplanted into cleared fat pads of recipient mice. GFP localization was analyzed by immunofluorescence in both endogenous Sca-$1^{+/GFP}$ tissue (C&D) and in transplanted tissue (A&B) (GFP and phalloidin–Texas Red). A confocal composite image (80 x 0.8μm sections) (A) and a single confocal image (0.8μm section) (B) from the same region illustrate intense GFP expression at the distal tips of growing ducts, as well as less intense expression in the mature ducts. A cross-section of ductal epithelium shows GFP expression detected sporadically in luminal epithelial cells (C). The abundance of GFP-expressing cells correlates with about 20% Sca-1^{pos} cells detected by FACS analysis (Fig. 2B and Fig. 4A). Three-dimensional rotation of this confocal image reveals GFP-expressing cells in contact with the lumen and others located basally. Additionally, GFP immunofluorescence does not colocalize with PR expression in luminal epithelial cells (D). FACS analysis reveals that Sca-1-expressing cells do not bind to peanut

lectin, a marker of differentiation in the mammary gland (E). Scale bars indicate 50 μm (A,B,D) and 10 μm (C).

Figure 4. Enriching Sca-1^{pos} Cells by Magnetic Sorting

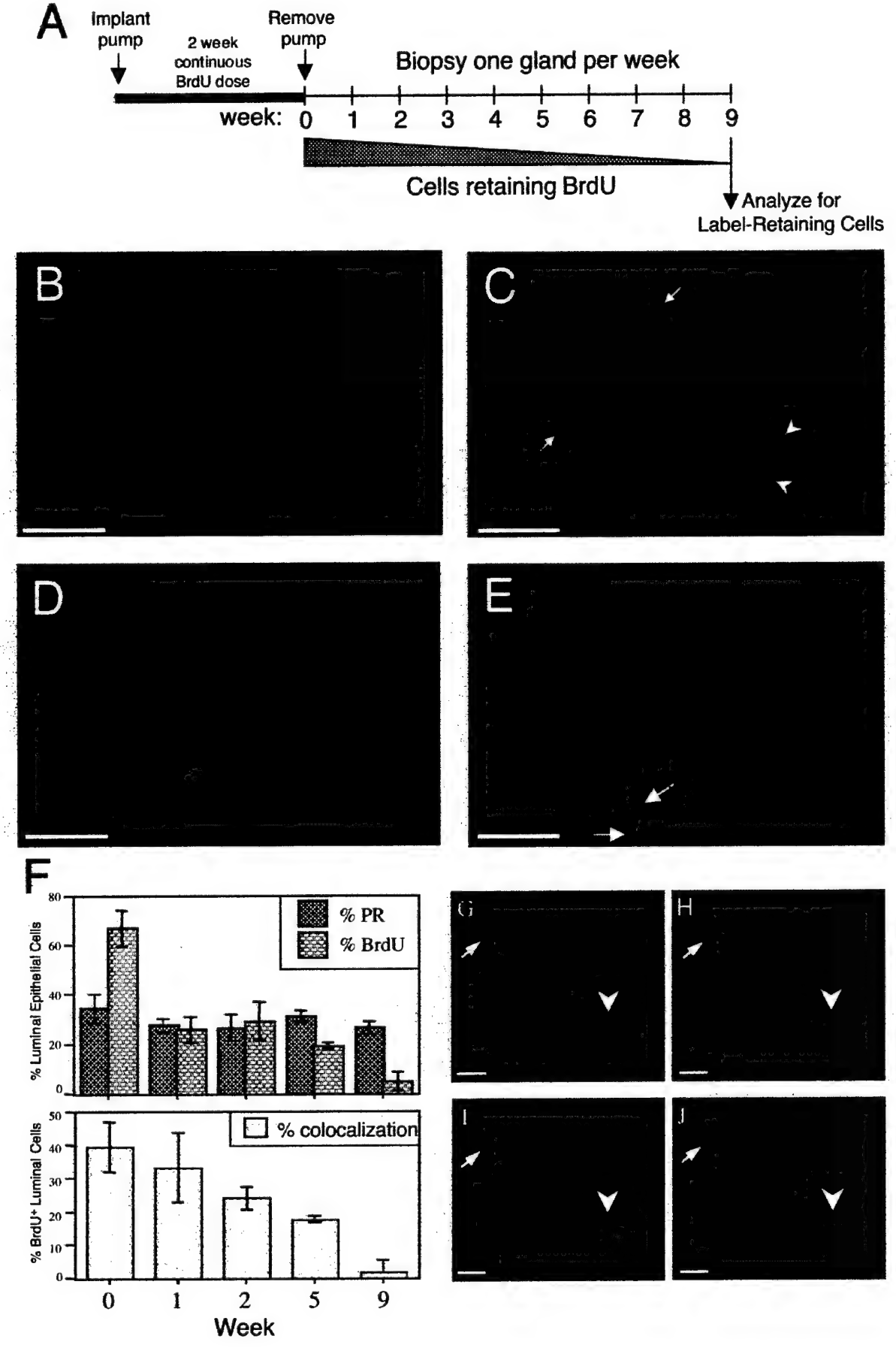
Sca-1^{pos} cells were isolated from total mammary epithelial cells by immunosorting using biotinylated anti-Sca-1 antibodies and streptavidin-conjugated microbeads. The results of a single round of enrichment are illustrated in (A-C): whole mammary epithelial cell fraction (A), Sca-1-depleted fraction (B), and Sca-1-enriched fraction (C). When these fractions were analyzed for LRCs, the Sca-1-enriched fraction contained a two-fold enrichment of LRCs compared to the Sca-1-depleted fraction (D).

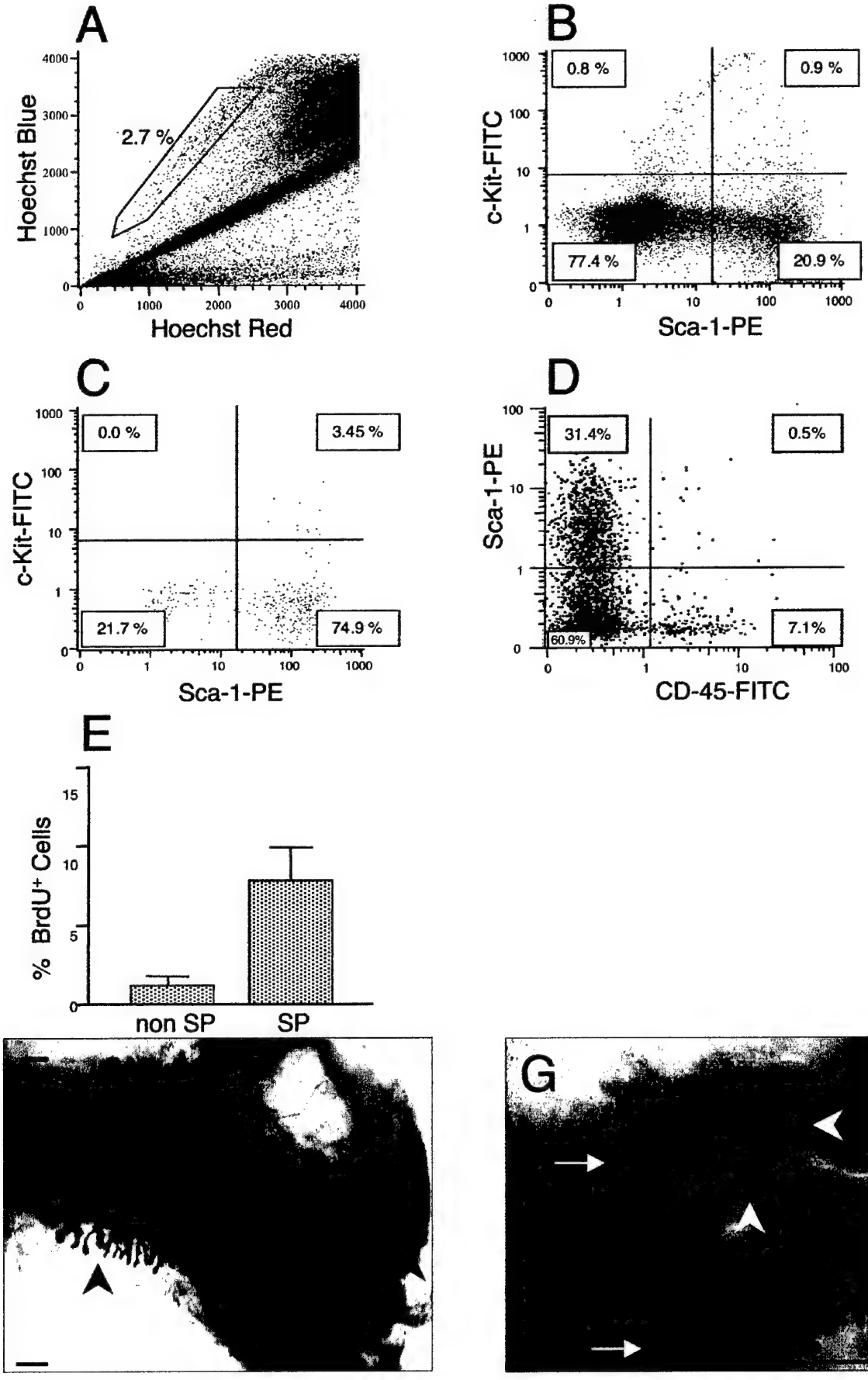
Figure 5. Outgrowth Potential of Sca-1-Sorted MECs

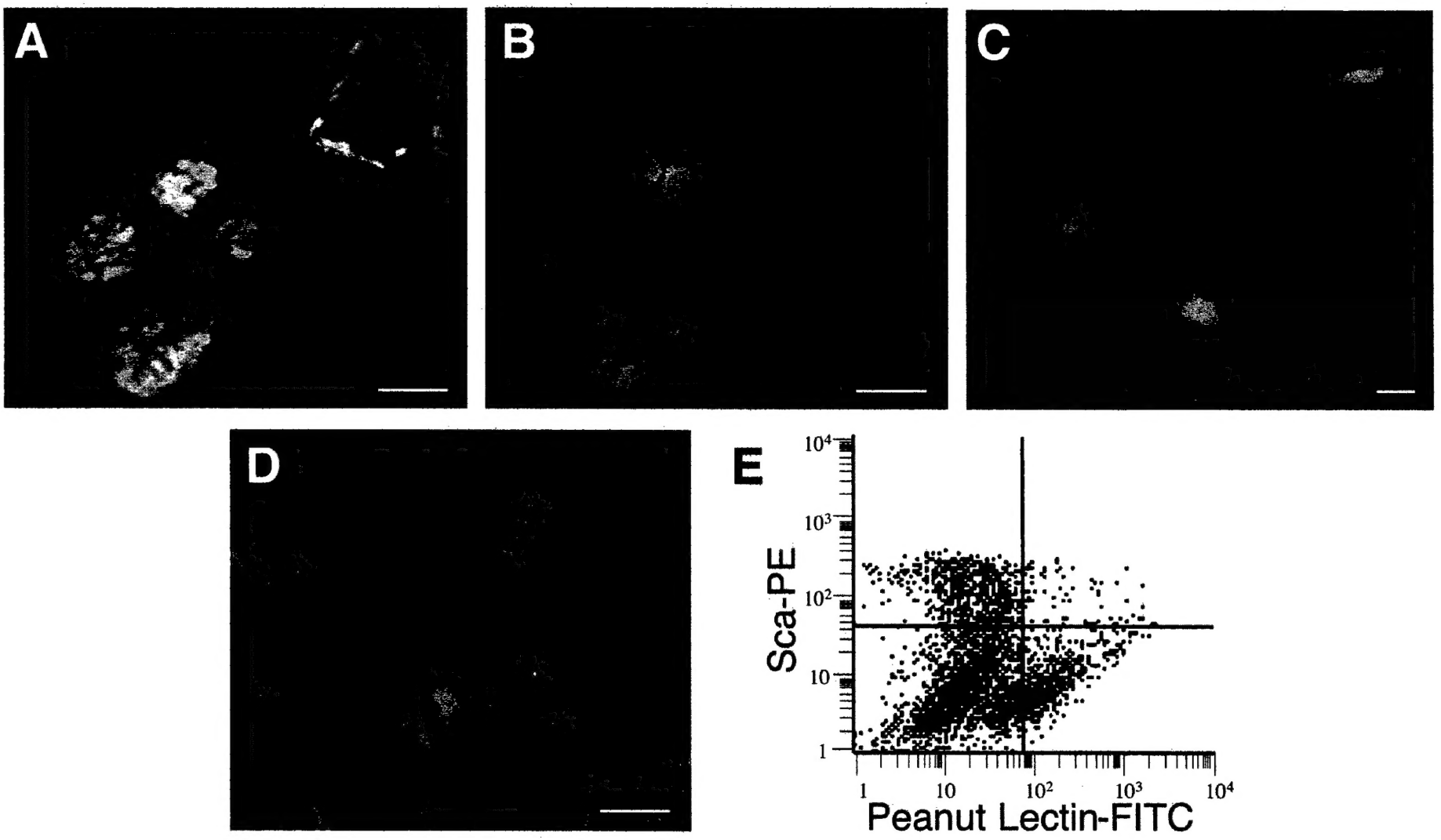
Using two methods of sorting, mammary epithelial cells were enriched for or depleted of Sca-1-expressing cells and were injected into cleared fat pads in a standard mammary reconstitution assay. Outgrowths were harvested after 6 weeks and the epithelium in the whole mounts was visualized by hematoxylin (B,C). Panel A outlines the sorting methods, purity of the sort, number of cells injected, and the extent of epithelial outgrowth. Injected cells formed either no epithelial structure (denoted as "–"), partial outgrowth or full outgrowth; partial outgrowth was defined as 5-50% of the fat pad filled with epithelial ductal structures (B) while full outgrowth was defined as >50% of the fat pad filled (C). (D-F) Normal ductal and alveolar development was detected in outgrowths derived from GFP^{pos} cells. Whole mounts (D) and H&E stained sections (E, F) of outgrowths derived from GFP^{pos} cells showed luminal, myoepithelial (F, arrowheads) and TEBs (E) with normal cap (arrowhead) and body (arrow) cell layers. Alveolar buds were also observed in these outgrowths in early pregnant mice (D,F, arrows). Scale bars indicate 1 mm.

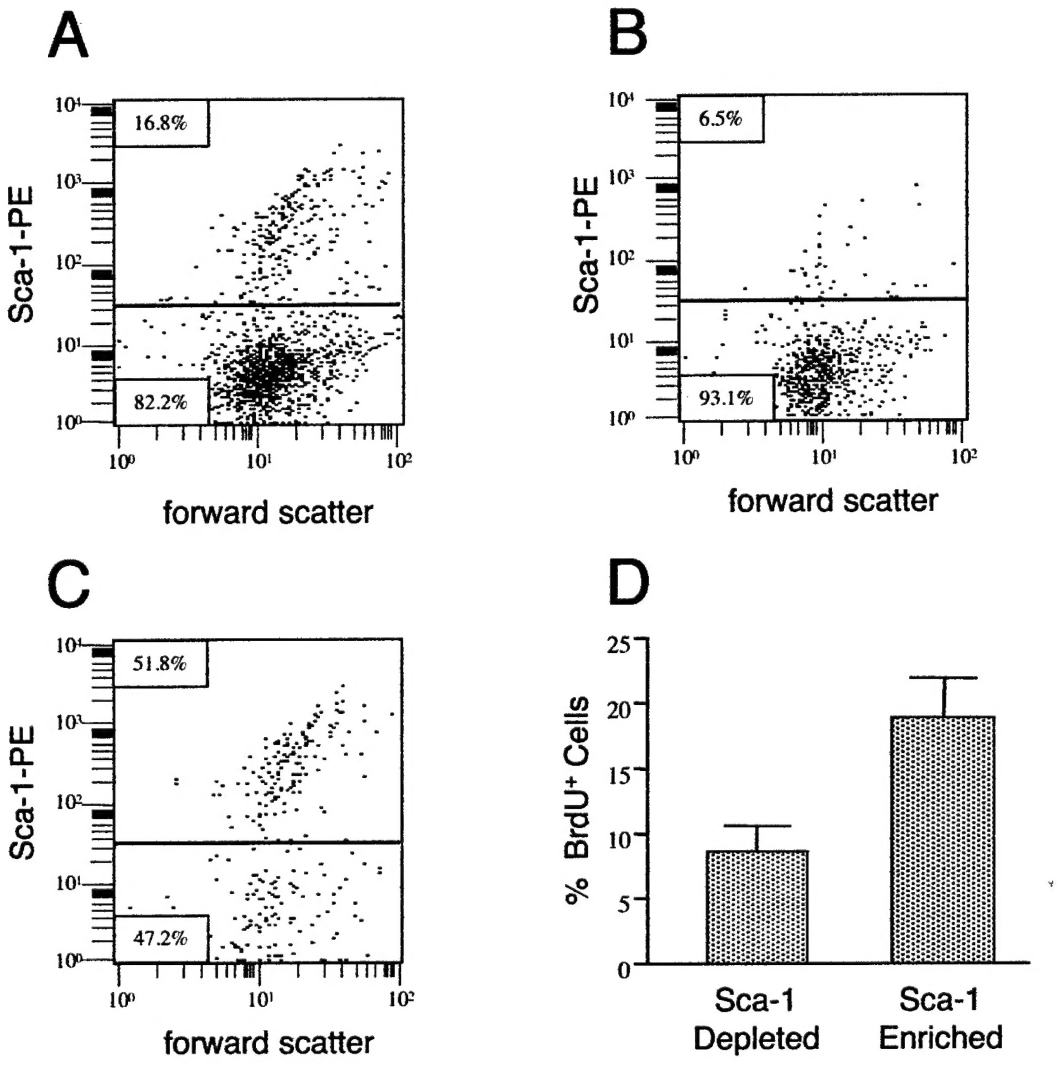
Figure 6. Model of MEC Progenitors

Mammary epithelium can be classified into several distinct cell populations including MG-SP, Sca-1^{pos}, and PR^{pos} cells. In this model, these populations may represent various stages of epithelial differentiation. LRCs are found primarily in the Sca-1 and MG-SP populations suggesting that these groups contain within them a quiescent sub-population. The MG-SP population may represent the most primitive and least differentiated subclass and may contain the multipotent stem cell population.


A
Implant pump
2 week continuous BrdU dose
Remove pump
Biopsy one gland per week
week: 0 1 2 3 4 5 6 7 8 9
Cells retaining BrdU
Analyze for Label-Retaining Cells
B
C
D
E
F
% Luminal Epithelial Cells
% PR
% BrdU
% BrdU+ Luminal Cells
% colocalization
0 1 2 5 9
Week
G
H
I
J

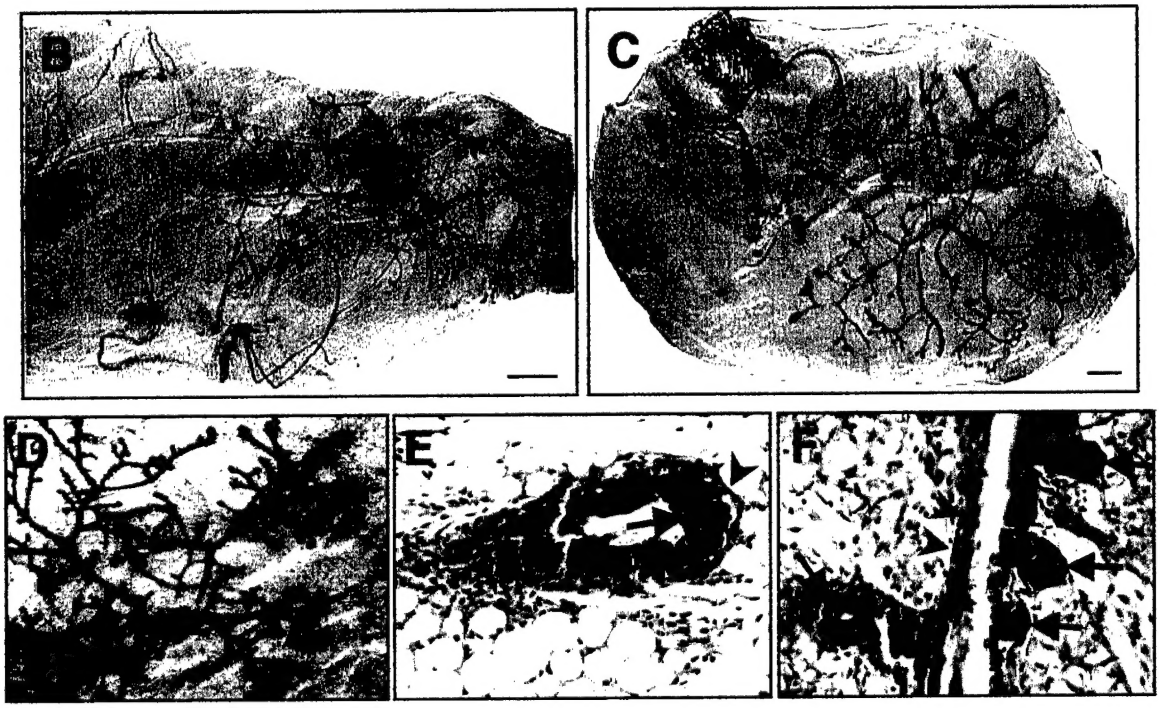

A
Hoechst Blue
Hoechst Red
2.7 %
B
c-Kit-FITC
Sca-1-PE
0.8 %
0.9 %
77.4 %
20.9 %
C
c-Kit-FITC
Sca-1-PE
0.0 %
3.45 %
21.7 %
74.9 %
D
Sca-1-PE
CD-45-FITC
31.4%
0.5%
60.9%
7.1%
E
% BrdU+ Cells
non SP
SP
G

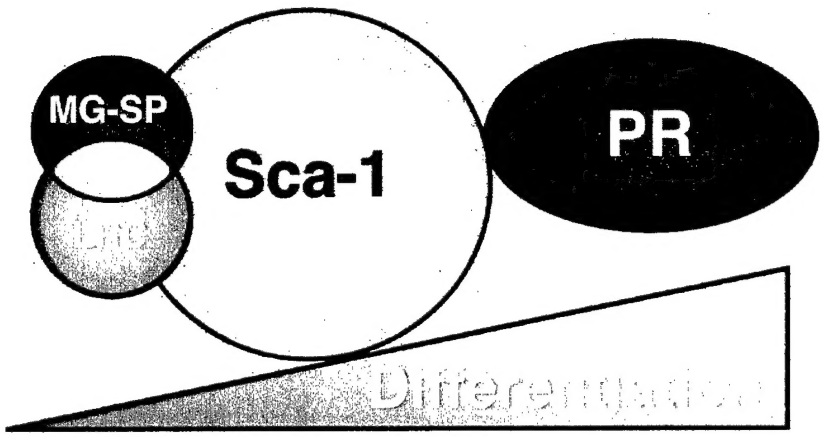

A
B
C
D
E
Sca-PE
Peanut Lectin-FITC
10^4
10^3
10^2
10
1
1
10
10^2
10^3
10^4


A
B
C
D
Sca-1-PE
forward scatter
16.8%
82.2%
6.5%
93.1%
51.8%
47.2%
% BrdU+ Cells
Sca-1 Depleted
Sca-1 Enriched

A

Enrichment Method	Sca-1 Positive			Sca-1 Negative		
	Cell No.	Outgrowth	Purity	Cell No.	Outgrowth	Purity
Magnetic Bead Sorting	10,000	Full	86%	10,000	–	92%
	10,000	Partial		10,000	Partial	
	10,000	Partial		10,000	Full	
	10,000	Partial		10,000	–	
	50,000	Full		50,000	–	
	50,000	Full		50,000	Partial	
	50,000	Full		50,000	Full	
	50,000	Full		50,000	–	
	50,000	Partial		50,000	–	
	50,000	Partial		50,000	–	
Sca-1-GFP	1000	Partial	91%	1000	–	99%
	2000	Full		2000	–	
	2000	Partial		2000	–	
	5000	Full		5000	–	
	5000	Partial		5000	–	
	10,000	Full		10,000	–	




MG-SP
Sca-1
PR
Differentiation